Role of *Staphylococcus Aureus* Surface-Associated Proteins in the Attachment to Cultured HaCaT Keratinocytes in a New Adhesion Assay

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Colonization of human skin with *Staphylococcus aureus* is a common feature in a variety of dermatologic diseases. In order to reproducibly investigate the adherence of *Staphylococcus aureus* to human epidermal cells, an *in vitro* assay was established using the biotin/streptavidine labeling system and the HaCaT cell line. This assay was used to define the role of several *Staphylococcus aureus* surface proteins with regard to their function in the staphylococcal adhesion process. Our studies included the standard laboratory strain *Newman* as well as its genetically constructed mutants DU5873, DU5852, DU5854, and DU5886 generated by allele replacement or transposon mutagenesis, which are deficient in the elaboration of staphylococcal protein A (spa), clumping factor (clfA), coagulase (coa), and the fibronectin-binding proteins A and B (fnbA/B), respectively. In comparison with strain *Newman* all mutants showed remarkably reduced adherence to the HaCaT keratinocyte cell line in our assay, yielding only between 43% and 60% of the adherence capacity of strain *Newman* after 60 min. Bacterial adherence could be re-established by introducing the cloned wild-type genes for the surface proteins on shuttle plasmids into the chromosomally defective mutants, thus suggesting a pathogenetic role of these proteins in the attachment of *Staphylococcus aureus* to human keratinocytes. Bacterial adherence was additionally enhanced by alkaline pH-values that are characteristic for skin conditions with epidermal barrier dysfunction. The use of *Staphylococcus aureus* mutant strains, deficient in the elaboration of defined proteins, allows specific investigation of colonization and virulence factors of this dermatologic relevant microorganism. *Key word: surface proteins. J Invest Dermatol 111:452–456, 1998*

Colonization of the skin with *Staphylococcus aureus* plays an important role in the pathogenesis of a variety of dermatologic diseases such as atopic dermatitis, psoriasis, and cutaneous T cell lymphoma (Aly *et al*, 1977; Abeck and Ruzicka, 1992; Nickoloff *et al*, 1993; Cooper, 1994; Tokura *et al*, 1995). Well-established virulence factors of these bacteria include their encymatic repertoire (Kornblum *et al*, 1990), their ability to trigger chronic inflammatory processes via the elaboration of superantigens (Tokura *et al*, 1994; Leung *et al*, 1995), and their widespread resistance to commonly used anti-microbial agents (Chambers and Hackbarth, 1989). Although numerous reports dealt with their pathogenetic properties, little is known about colonization factors of *S. aureus* to human skin.

Staphylococcus aureus surface proteins have been supposed to play a major role in the adhesion process and a previous report established the influence of Protein A on the bacterial adherence to isolated human corneocytes (Cole and Silverberg, 1986). Further investigations gave evidence for an important role of the fibronectin receptor in the colonizing process of low granular and fully keratinized cells (Bibel *et al*, 1983).

In the meantime, additional *S. aureus* structures have been identified enabling this microorganism to gain access to different types of human tissue (Lopes *et al*, 1985; Jönsson *et al*, 1991; Liang *et al*, 1993; Foster and McDevitt, 1994; Kanzaki *et al*, 1996; Foster *et al*, 1997). Among these are coagulase and clumping factor (Phonimdaeng *et al*, 1990; McDevitt *et al*, 1992, 1994).

Genetic manipulation of the well-characterized strain *Newman* allows the construction of stable mutants of this strain deficient in the expression of Protein A, coagulase, clumping factor, and fibronectin-binding protein A and B (Patel *et al*, 1987; Phonimdaeng *et al*, 1990; McDevitt *et al*, 1994; Greene *et al*, 1995). To investigate their capacity to adhere to human keratinocytes, we established an *in vitro* adherence assay that allows the quantitation of adherent bacteria using the biotin/streptavidine labeling system. In addition the influence of pH values and temperature on bacterial adherence was investigated.

MATERIAL AND METHODS

Cell culture and media The HaCaT keratinocyte cell line that was generously provided by Prof. Fusenig (DKFZ, Heidelberg, Germany) was used throughout the study. Cells were grown in Dulbecco's keratinocyte SF medium (Gibco, Grand Island, NY) with the addition of epidermal growth factor and bovine pituary extract (Gibco) at 37°C and 5% CO_2. No fetal calf serum was added to the medium. Cells were grown in 96 well plates (Nunc, Roskilde, Denmark) until confluent monolayers were obtained. Repeated counts of the keratinocytes in a Neubauer counting chamber at that point revealed a

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Abbreviation: CFU, colony forming unit.

Table I. Bacterial strains used in the study

S. aureus strain	Relevant genotype	Clumping	IgG-binding (OD)	Coagulase titres	Fibronectin binding (OD)
Newman Parent strain	*coa*$^+$, *clf* $^+$, *spa*$^+$, *fnbA/B*$^+$	+	2.341	>1:128	2.147
DU5854 Coagulase-deficient	Δ*coa::Tc*^{r}	+	2.316	1:8	2.052
DU5852 Clumping factor-deficient	*clfA1::Tn*917	–	2.472	>1:128	1.786
DU5873 Protein A-deficient	Δ*spa::Tc*^{R}	+	0.232	>1:128	2.011
DU5886 Fibronectin-binding protein A/B-deficient	*fnbA::Tc*^{R}; *fnbB::Em*^{R}	+	2.186	>1:128	0.566
DU5854/p^{coa} Coagulase-restored	Δ*coa::Tc*^{R}/*pcoa*$^+$	+	2.250	>1:128	2.002
DU5852/p^{clf} Clumping factor-restored	*clfA1::Tn*917/*pclf*$^+$	+	2.017	>1:128	2.201
DU5873/p^{spa} Protein A-restored	Δ*spa::Tc*^{R}/*pspa*$^+$	+	1.989	>1:128	1.989
DU5886/p^{fnbA} Fibronectin-binding protein A-restored	*fnbA::Tc*^{R}; *fnbB::Em*^{R}/*pfnbA*$^+$	+	2.004	>1:128	1.967
DU5886/p^{fnbB} Fibronectin-binding protein B-restored	Δ*fnbA::Tc*^{R}; Δ*fnbB::Em*^{R}/*pfnbB*$^+$	+	2.183	>1:128	2.111

concentration of 1.5×10^4 cells per well. In order to minimize unspecific adhesion the keratinocyte plates were blocked for 120 min with 2% bovine serum albumin (Boehringer, Mannheim, Germany) in phosphate-buffered saline (PBS) before starting the assay.

Bacterial strains *Staphylococcus aureus* strain *Newman* is a standard strain and has been used in previous studies (Cole and Silverberg, 1986; Moreillon *et al*, 1995).

The following genetically manipulated strains were used for the determination of staphylococcal adhesion structures: strain DU5852 is a clumping factor negative mutant that was constructed by transposon *Tn917* mutagenesis of strain *Newman*, forming *clfA1::Tn917* (McDevitt *et al*, 1994). Strain DU5854 is a coagulase-defective mutant of strain *Newman* with a deletion in the *coa* gene substituted by a fragment encoding tetracycline resistance (Δ*coa::Tc*^{R}) (McDevitt *et al*, 1992). Strain DU5886 is strain *Newman* carrying mutations in the fibronectin-binding proteins A + B, marked by a tetracycline resistance in the fibronectin-binding protein A (*fnbA::Tc*^{R}) and a erythromycin resistance in the fibronectin-binding protein B (*fnbB::Em*^{R}) (Greene *et al*, 1995). Strain DU5873 is a Protein A-negative *Newman* mutant with a tetracycline resistance in the spa gene (Δ*spa::Tc*^{R}) (Patel *et al*, 1987).

In order to verify the role of the relevant surface-protein genes the wild-type gene was restored in all mutants using staphylococcal shuttle plasmids in accordance to previously described reports (Greene *et al*, 1995). **Table I** gives a summary of the strains and their relevant phenotype.

Detection of protein A For the detection of staphylococcal protein A we used a method described by van Belkum *et al* (1997) quantifying this protein by its capacity to bind to purified IgG. For this test, overnight cultures of the strains were grown in tryptic soy broth (Gibco) and diluted to an OD_{570} of 0.1. One hundred microliters of this suspension were used for coating Nunc Immunoplates (Nunc). After 1 h at 20°C the plates were washed three times with PBS. Remaining binding sites were blocked with 2% bovine serum albumin (Boehringer) in PBS. After two washes with PBS, 50 μl of alkaline-phosphatase coupled rabbit anti-mouse immunoglobulin (Sigma, Munich, Germany) diluted in PBS were applied to the wells and incubated for 1 h at 20°C. After three washes with PBS, alkaline phosphatase substrate (Sigma) was added to the wells and allowed to interact for 30 min. The obtained optical density was read at 492 nm. All strains were tested in quadruplicate.

Detection of coagulase Coagulase titres were measured by adding 0.5 ml of dilutions of staphylococcal culture supernatans to 0.5 ml of human plasma diluted 1:3 in PBS. The titer was the reciprocal of the highest dilution showing evidence for clotting after 24 h incubation at 37°C (Anderson *et al*, 1982).

Detection of fibronectin-binding proteins Fibronectin binding was tested by screening their capacity to adhere to bovine fibronectin-coated 96 well culture plates (Becton-Dickinson, Heidelberg, Germany). Therefore the strains were grown overnight in tryptic soy broth (Gibco) at 37°C. Biotin-NHS (Sigma) was added at a concentration of 5 mM to the growth medium and was allowed to interact with the bacteria for 90 min at 37°C. Bacteria were harvested by 10 min of centrifugation at 3000 r.p.m. and resuspended in PBS. In order to remove unbound biotin from the suspension this washing procedure was repeated three times. The pelleted bacteria were diluted in PBS to an optical density of 0.1 at 570 nm. Two hundred microliters of this dilution were applied to each well of the fibronectin coated plates and were incubated at 37°C for 1 h. After three washes with PBS, 50 μl alkaline phosphatase coupled streptavidine at a concentration of 250 ng per ml (Sigma) was added and incubated for 30 min, followed by another three washes. Thereafter 50 μl of alkaline phosphatase substrate (Sigma) was added to the wells and the reaction was stopped with 3 M NaOH after 30 min. Extinction was read at 405 nm. Strains were tested in triplicate.

Detection of clumping factor Measurement of cell clumping was detected using purified fibrinogen (Sigma) in the slide agglutination test.

Adhesion assay Bacteria were grown in tryptic soy broth (Gibco) into early logarithmic growth phase (≈4 h) at 37°C with gentle shaking. Labeling of the bacteria with biotin was carried out in modification of a previously described method (Tompkins *et al*, 1990; von Boxberg *et al*, 1990). In brief, biotin-NHS (Sigma) was added at a concentration of 5 mM to the growth medium and was allowed to interact with the bacteria for 90 min at 37°C. Bacteria were harvested by 10 min of centrifugation at 3000 r.p.m. and resuspended in PBS. In order to remove unbound biotin from the suspension this washing procedure was repeated three times. The pelleted bacteria were diluted in PBS and the optical density (OD) was controlled in a spectrophotometer at 570 nm.

One hundred microliters of the bacterial suspension was added to each confluent keratinocyte well and the plates were incubated at 37°C. After various time points the plates were washed three times with PBS and 100 μl of streptavidine coupled with alkaline phosphatase (Sigma) at a concentration of 250 ng per ml was added to each well. After 30 min of incubation at 37°C 100 μl alkaline phosphatase substrat (Sigma) was added and was allowed to interact with the enzyme for 30 min before the reaction was stopped with the addition of 50 μl 3 M NaOH. Optical density (OD) was measured at a wavelength of 405 nm. All strains were tested in quadruplicate.

Internal controls were used as follows: to rule out unspecific binding of the bacteria to the culture plates, appropriate dilutions of the bacterial suspension were incubated on an empty row of the plates and treated in the same manner as the keratinocyte-coated rows. The obtained OD was considered unspecific and was substracted from the experiment results as background value.

To control for comparable labeling of the different strains with biotin, the labeled strains were diluted (OD_{570} 0.1) in PBS and 100 μl of the dilution were exposed to 100 μl of several concentrations (25 ng per ml, 250 ng per ml, and 2.5 μg per ml) of the alkaline phosphatase/streptavidine complex for 15 min at 37°C and were then allowed to interact with 50 μl of the alkaline phosphatase substrat for 30 min. Strains were only considered equally labeled when the photometric evaluation (OD_{405}) of this dilutions yielded comparable results. The possibility of endogenous phosphatase activity of the tested strains influencing the results of the experiment was ruled out by incubating 4×10^8 bacteria, diluted in PBS (OD_{570} 0.5) with the alkaline phosphatase substrate for 60 min. This control showed negative results for all tested strains. Binding

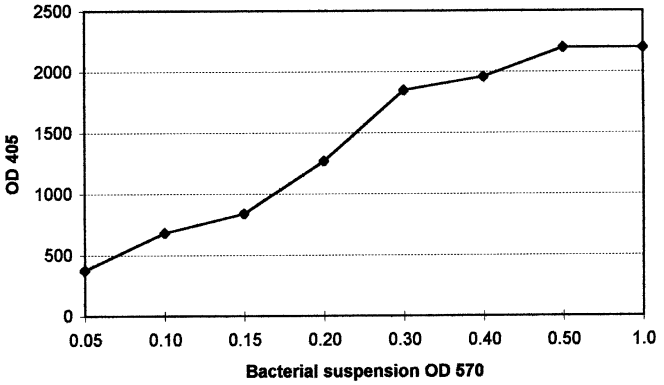

Figure 1. Concentration dependency of incubated bacteria. Strain *Newman* was diluted in PBS obtaining varying optical densities at 570 nm. OD 0.2 was chosen for further experiments as this dilution allowed the best discrimination. One milliliter of this dilution contained 5×10^6 CFU by counting on Columbia agar.

of streptavidine to the keratinocytes was separately tested and revealed no positive results.

The obtained results of the adhesion assay in the dose defining experiment were verified with the colony forming units (CFU) count. For this procedure the bacteria/keratinocytes complex was lifted with trypsin-ethylenediamine tetraacetic acid (Sigma) from the plates and was diluted in distilled water to rupture the keratinocytes. Dilutions of the bacteria were plated on Columbia agar from BioMerieux (France) containing 5% sheep blood. After 24 h at 37°C the CFU were counted.

Immunohistochemistry Cells were grown on sterile glass slides in cell culture dishes in SFB medium until confluency was obtained. Slides were fixed in acetone and immunohistochemistry was carried out following standard protocols using the APAAP-technique (Dako, Hamburg, Germany). The anti-fibronectin antibody was obtained from Sigma and used in a 1:500 dilution in PBS. Keratinocytes were counterstained in Mayer's Hematoxylin (Merck, Darmstadt, Germany) and staphylococci were stained with gentian violet in a modified Gram stain.

RESULTS

Evaluation of mutant strains The *S. aureus* mutant strains were controled for lack of elaboration of the deleted gene products by a battery of tests. **Table I** gives a summary of the specific results. Interestingly, despite the deletion of the fibronectin-binding protein as well as the Protein A encoding gene sequences, we still obtained a rest of binding to the fibronectin and IgG-coated wells, respectively. This result may represent a nonreceptor/ligand-specific attachment to these molecules.

Concentration dependency of incubated bacteria In order to define an adequate keratinocyte/bacteria ratio, the parent strain *Newman* was used in several dilutions. As shown in **Fig 1** an OD_{570nm} of 0.2 turned out to allow a good evaluation of *S. aureus* adherence. Bacterial counts at that OD revealed a number of 5×10^6 CFU per milliliter. This dilution was used throughout the following experiments.

Counts for the adherent bacteria yielded results between 0.7×10^3 CFU per ml at OD_{570} 0.05 and 0.9×10^8 CFU at OD_{570} 1.0 on Columbia agar.

Influence of pH, temperature, and EGF on bacterial adherence Changes of the skin surface pH are known to influence the microbial colonization process. Therefore we screened the parent strain *Newman* for its adhesion properties at different pH values. This experiment showed optimal cell adherence at a slightly alkaline pH value between 7 and 8 (**Fig 2**). This pH was kept for testing the adherence of the mutant strains. Varying the incubation temperature resulted in highest adherence at 37°C (**Fig 3**), which was also used in the following experiments. Withdrawing the keratinocytes from epidermal growth factor for 24 h resulted in a clear decrease of adherence, possibly due to the diminished number of ligand structures on the keratinocye surface (data not shown).

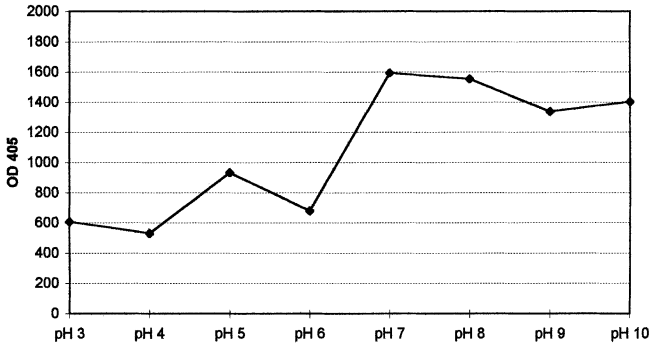

Figure 2. Influence of pH on bacterial adherence. Adherence capacities of strain *Newman* were taken at different pH values. Alkaline pH showed better adhesion results with an optimum between pH 7 and pH 8.

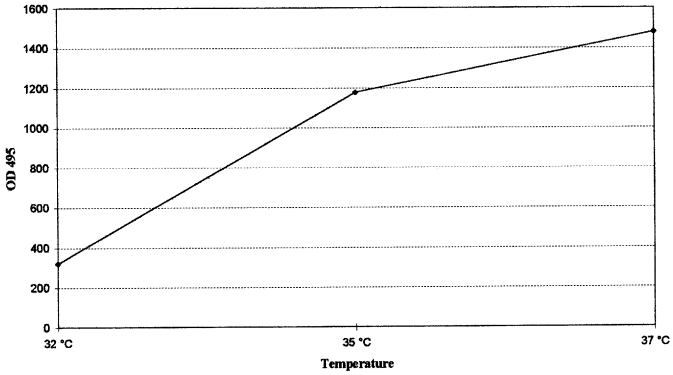

Figure 3. Influence of temperature on staphylococcal adherence. Strains were grown at different temperatures in tryptic soy broth until identical turbidities of the cultures were obtained and bacteria were incubated with the keratinocytes. Growth at 37°C was faster as compared with 32°C and 35°C. Attachment capacities were increased at higher temperatures.

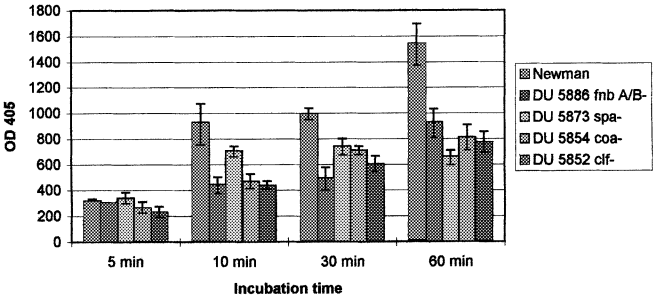

Figure 4. Adherence features of mutant strains. Adherence testing (±SEM) of the mutants revealed clearly diminished adherence capacities for the surface protein-deficient mutants, yielding 53% of the parent strain *Newman* adherence for the clumping factor-negative mutant DU5852, 50% for the coagulase-negative mutant DU5854, and 60% for the fibronectin binding-protein A/B-negative mutant DU5886. The lowest level was obtained for Protein A-negative mutant DU5873 with 43% of strain *Newman*.

Adherence of *S. aureus* mutant strains Testing the *S. aureus* mutant strains showed a decrease in the attachment to the HaCaT cells. As shown in **Fig 4**, the coagulase-negative mutant DU5854 showed only 53% of the adherence capacity of parent strain *Newman*, the clumping factor-deficient mutant DU5852 reached 50%, the fibronectin-binding-protein A and B-negative mutant DU5886 was reduced to 60% of the parent strain, and the Protein A-negative strain DU5873 reached 43%. Strains were read after 60 min. The results for DU5852 and DU5873 hit the criteria for statistical significance using the Student's t test ($p < 0.05$), whereas the results for DU5854 and DU5886 remained sharply above the cut off for significance ($p = 0.058$ and $p = 0.055$, respectively).

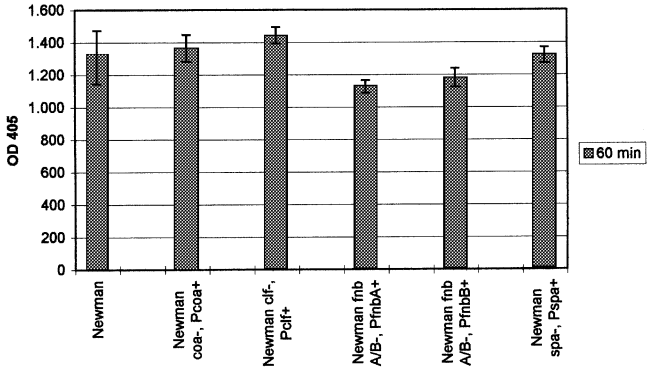

Figure 5. Adherence of wild-type restored mutants. Restoring the wild-type genes for the deleted genes on shuttle plasmids resulted in an adherence pattern that resembled the parent strain *Newman*. The results for the restored mutants fnbA+ and fnbB+ stayed slightly below strain *Newman*, whereas adherence for the clumping factor and the coagulase restored wild-type showed even higher levels, probably due to a plasmid-multi copy effect.

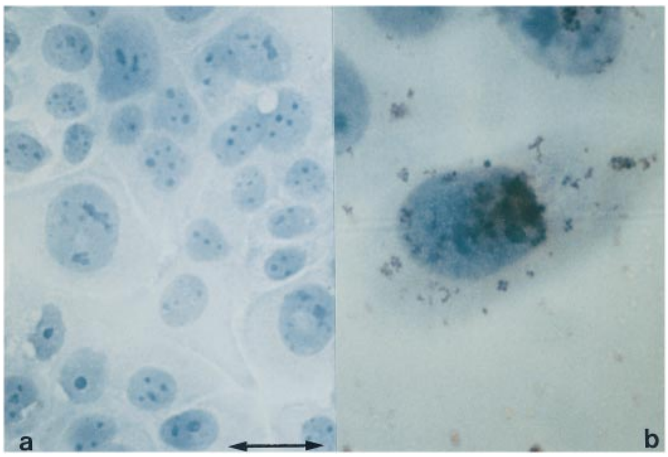

Figure 6. Immunostaining of fibronectin on HaCaT keratinocytes in the presence of *S. aureus*. (*a*) Monolayer of the HaCaT keratinocyte stained with hematoxylin. (*b*) Distribution of fibronectin stained with a monoclonal antibody (red structures). Strain *Newman* adheres to the matrix protein. Bacteria are stained with gentian violet and adhere in clusters of grapes to the keratinocyte. As seen in some parts of the cell, attachment is not limited to fibronectin suggesting further relevant cellular ligands for *S. aureus*. Bacteria were incubated for 60 min with the keratinocytes. *Scale bar*: 10 μm.

Adherence of wild-type gene restored mutants Restoring the wild-type gene of the knock-out mutants via the introduction of a shuttle plasmid carrying the responsible gene resulted in an overall regain of the adherence capacities of the strains, with the exception of the fibronectin-binding protein A and B negative double mutant that yielded statistically not significant lower adherence when complemented with either Fn-binding protein A or Fn-binding protein B. As shown in **Fig 5**, restoring the coagulase negative mutant resulted in 103%, restoring the clumping-factor-negative mutant resulted in 109%, and restoring the protein A-negative mutant resulted in 96% of the parental adherence. The fnb A and B double mutant yielded 85% when complemented with fnb A and 89% when complemented with fnb B.

Expression of fibronectin in keratinocytes-*S. aureus* interaction To demonstrate the expression of fibronectin as a matrix protein and well-known staphylococcal adhesion factor, we stained for fibronectin with a monoclonal antifibronectin antibody in the presence of strain *Newman*. **Figure 6(*b*)** shows adhesion of the staphylococci to the marked matrix protein and, although to a lesser extent, to additional keratinocyte structures.

DISCUSSION

Attachment to and colonization of the human skin by *S. aureus* has previously been reported to play a crucial role in the pathogenesis and the perpetuation of several dermatologic diseases (Aly *et al*, 1977; Abeck and Ruzicka, 1992; Nickoloff *et al*, 1993; Cooper, 1994; Tokura *et al*, 1995). Quantitation of this process in recent studies was mainly based on counting the bacteria adherent to the single epidermal cell types microscopically (Cole and Silverberg, 1986). This technique, however, unfortunately lacks the possibility to test a defined layer of cells concerning the bilateral expression of adhesins.

The assay described here provides a rapid and reproducible method for the quantitation of staphylococcal adherence factors to cultured human keratinocytes using the biotin/streptavidine system. In addition, the investigation of genetically engineered mutants of the pathogenetic *S. aureus* strain *Newman* allows the identification of bacterial structures playing a role in the colonization process by disabling the bacteria in their expression of defined surface proteins. Already previous studies suggested specific receptor/ligand binding as a major adhesion factor for *S. aureus* to keratinocytes and a variety of structures have been supposed as a specific counterpart for the staphylococcal protein A (Bibel *et al*, 1983; Cole and Silverberg, 1986). To approach this question we used various genetically defined mutants of strain *Newman* that have been generated in a battery of experiments dealing with the colonization of *S. aureus* to various human tissues (Patel *et al*, 1987; Phonimdaeng *et al*, 1990; McDevitt *et al*, 1994; Greene *et al*, 1995). To some extent we could find parallels to keratinocytes.

The important role of Protein A for the staphylococcal adhesion to human epidermal cells has been previously described (Cole and Silverberg, 1986). This result was confirmed by the low adhesion capacities of Protein A-negative mutant DU5873 to the cultured monolayers. Although its counterpart on human keratinocytes and corneocytes has still not been identified so far, the strong affinity of Protein A for the Fc-terminus of human IgG-antibodies in inflamed tissue might enable *S. aureus* to gain access to the human epidermis. As with our model the presence of such antibodies could be ruled out, a second, maybe not receptor-specific adhesion mechanism has to be considered.

The diminished adherence of strain DU5886 impaired in the elaboration of the fibronectin-binding proteins A and B is consistent with the findings of Bibel *et al* (1983), who previously described an essential role for this matrix protein in the adhesion process by using purified fibronectin. Their finding that Protein A is not the ligand for human fibronectin has been supported by the identification of the genes responsible for the staphylococcal fibronectin-binding protein A and B (Jönsson *et al*, 1991). This group could show that two different genes separated by a stretch of 682 bp on the chromosome of *S. aureus* are responsible for fibronectin binding. Important work was added by Greene *et al* (1995), who demonstrated that deletion in only one of these genes did not lead to a significant decrease in the binding of *S. aureus* to fibronectin-coated coverslips, whereas the double mutant almost completely lost its binding capacity. Restoring either one of the fibronectin-binding proteins on shuttle plasmids resulted in full adhesion to the coverslips. As a consequence of these findings we used the double mutant DU5886 in our assay. The decreased adherence of this mutant to the keratinocytes could be partially restored when either a fnbA- or a fnbB-carrying plasmid was introduced into DU5886, although neither restoration resulted in complete regain of adherence; however, these results failed statistical significance. These findings confirm an important role for human fibronectin as ligand for *S. aureus* on human keratinocytes, as further confirmed by the immunohistochemical colocalization in the photomicrographs.

Investigating the coagulase-negative and clumping factor-negative variants DU5852 and DU5854 revealed some interesting findings. Although the main ligand for these virulence factors is represented by the plasma protein fibrinogen (McDevitt *et al*, 1992; Dickinson *et al*, 1995; Moreillon *et al*, 1995; Vaudaux *et al*, 1995), the defective mutants showed decreased adherence capacities to the cultured cells. Additionally to the serum fibrinogen binding, a second cellular-based adhesion mechanism has to be considered. In the clinical setting of chronically inflamed skin the action of these two staphylococcal virulence factors can be explained by the increased presence of fibrinogen on epidermal structures.

Introducing the wild-type plasmid into the clumping-factor defective

variant interestingly resulted in a slightly increased adherence of 109%. This phenomenon, however, might be the consequence of a multicopy effect that undergo bacterial plasmids when duplicated independently from the chromosomal duplication process.

These results demonstrate that more than one adhesin of *S. aureus* plays a role in the adhesion to the human epidermis. Recent findings in the molecular organization of the bacterial genome revealed important insights of the regulation of a variety of staphylococcal proteins. Regulating operons as *agr* and *sar* are able to control the expression of surface structures and enable this microorganism to increase the production of adhesins (Kornblum *et al*, 1990; Cheung and Projan, 1994, 1997).

In addition, environmental factors may also have an impact on bacterial colonization. One of these is the skin surface pH for which we could show an increased adherence of strain *Newman* at alkaline pH values, which are characteristic for diseases with epidermal barrier dysfunction such as atopic dermatitis. Changes in the pH on the other hand are known to have an impact on the expression of the above mentioned regulatory systems in *S. aureus* (Regassa *et al*, 1992).

As in recent years a variety of additional human matrix proteins have been identified to act as receptors for staphylococcal proteins such as fibronectin, vitronectin, and laminin (Lopes *et al*, 1985; Jönsson *et al*, 1991; Liang *et al*, 1993); further genetic studies should focus on *S. aureus* mutants defective in the respective binding proteins alone and in combination.

A possible therapeutic approach to prevent staphylococci to pathogenetically colonize the human skin could result in blocking the responsible structures.

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